PKHD1 Gene Silencing May Cause Cell Abnormal Proliferation through Modulation of Intracellular Calcium in Autosomal Recessive Polycystic Kidney Disease

Jiyun Yang1, Sizhong Zhang1,*, Qin Zhou1, Hong Guo2, Ke Zhang2, Rong Zheng2 and Cuiying Xiao1

1Department of Medical Genetics, West China Hospital, Sichuan University, Guoxue Xiang No. 37, Chengdu 610041, PR China
2Division of Human Morbid Genomics, State Key Laboratory of Biotherapy, Chengdu 610041, PR China

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Autosomal recessive polycystic kidney disease (ARPKD) is one of the important genetic disorders in pediatric practice. Mutation of the polycystic kidney and hepatic disease gene 1 (PKHD1) was identified as the cause of ARPKD. The gene encodes a 67-exon transcript for a large protein of 4074 amino acids termed fibrocystin, but its function remains unknown. The neoplastic-like in cystic epithelial proliferation and the epidermal growth factor/epidermal growth factor receptor (EGF/EGFR) axis overactivity are known as the most important characteristics of ARPKD. Since the misregulation of Ca\(^{2+}\) signaling may lead to aberrant structure and function of the collecting ducts in kidney of rat with ARPKD, present study aimed to investigate the further mechanisms of abnormal proliferation of cystic cells by inhibition of PKHD1 expression. For this, a stable PKHD1-silenced HEK-293T cell line was established. Then cell proliferation rates, intracellular Ca\(^{2+}\) concentration and extracellular signal-regulated kinase 1/2 (ERK1/2) activity were assessed after treatment with EGF, a calcium channel blocker and agonist, verapamil and Bay K8644. It was found that PKHD1-silenced HEK-293T cell lines were hyperproliferative to EGF stimulation. Also PKHD1-silencing lowered the intracellular Ca\(^{2+}\) and caused EGF-induced ERK1/2 overactivation in the cells. An increase of intracellular Ca\(^{2+}\) in PKHD1-silenced cells repressed the EGF-dependent ERK1/2 activation and the hyperproliferative response to EGF stimulation. Thus, inhibition of PKHD1 can cause EGF-induced excessive proliferation through decreasing intracellular Ca\(^{2+}\) resulting in EGF-induced ERK1/2 activation. Our results suggest that the loss of fibrocystin may lead to abnormal proliferation in kidney epithelial cells and cyst formation in ARPKD by modulation of intracellular Ca\(^{2+}\).

Keywords: Autosomal recessive polycystic kidney disease, Epidermal growth factor, Extracellular signal-regulated kinase, Intracellular calcium, PKHD1

Introduction

Autosomal recessive polycystic kidney disease (ARPKD) is an important renal and hepatic disease in neonates and infants, occurring 1 in 20,000-40,000 live births (Zerres et al., 1998). The main pathologic manifestations of the disease are the fusiform dilation of renal collecting ducts and distal tubules as well as dysgenesis of the hepatic portal triad including hyperplastic biliary ducts and congenital hepatic fibrosis (Roy et al., 1997; Zerres et al., 1998). In infancy, the disease results in significantly enlarged polycystic kidneys and pulmonary hypoplasia. Up to 30% of the affected neonates die of secondary respiratory failure shortly after birth (Zerres et al., 1994; Zerres et al., 1996). In subset that survived the perinatal period, the morbidity and mortality are mainly due to severe systemic hypertension, renal failure, and portal hypertension (Zerres et al., 1996; Roy et al., 1997; Fonck et al., 2001). Therefore, the patients with ARPKD are candidates for liver, kidney or combined liver and kidney transplantation (Sairanen et al., 1997).

In 1994, the gene for ARPKD was mapped to human chromosome 6p21-cen (Zerres et al., 1994; Zerres et al., 1994). Then independent groups isolated the gene (Xiong et al., 2002; Ward et al., 2002; Onuchic et al., 2002), and several disease-causing mutations in the PKHDI gene were identified (Bergmann et al., 2004; Bergmann et al., 2005). The gene encodes a novel large transmembrane protein with 4074...
amino acids named fibrocystin or polyductin (Ward et al., 2002; Onuchic et al., 2002). Studies have shown that its mouse gene homologue Pkhd1 is expressed in ductal structures during development of kidney, liver, lung, pancreas, and vessels (Nagasawa et al., 2002; Xiong et al., 2002). In adult kidney, Pkhd1 is expressed in the epithelia of proximal convoluted tubules, ascending limbs of Henle’s loop and collecting ducts. The protein is localized in the basal bodies and primary cilia of renal epithelia (Zhang et al., 2004).

Recently it has been found that fibrocystin interacts with calcium modulating cyclophilin ligand (CAML), a protein that is involved in Ca\(^{2+}\) signaling (Nagano et al., 2005), and the calcium influx into cells can change the gene expression and the control of cell growth and differentiation (Li et al., 2005; Kandili and Groszfeld, 2005; Kuipa et al., 2005). Meanwhile loss of cilia also may result in misregulation of Ca\(^{2+}\) and elevation of subapical membrane Ca\(^{2+}\). The defect in induced Ca\(^{2+}\) signaling leads to aberrant structure and function of the collect ducts in ARPKD (Liu et al., 2005; Siroky et al., 2006).

The most important characteristics of ARPKD development are neoplastic-like in cell proliferation and the EGFR axis overactivity (Richards et al., 1998; Veizis and Cotton, 2005). The phosphorylated ERK1/2 is significantly increased in cystic compared with noncystic kidneys and a sustained high level of MAPK signaling may influence cellular proliferation and function of cystic epithelial cells (Veizis and Cotton, 2005). Since abnormal epithelial cell proliferation underlies the cyst formation and renal enlargement in ARPKD, above findings suggest that activation of the MAPK pathway is critical in the development of the cystic changes. Therefore, it may be postulated that fibrocystin, as a membrane receptor, participates in EGF-induced abnormal proliferation of renal epithelial cells in ARPKD through intracellular Ca\(^{2+}\) to modulate the MAPK activity. To test this idea, we tried to determine whether the inhibition of Pkhd1 expression by gene silencing could increase EGF-induced cell proliferation and its possible mechanisms.

Materials and Methods

Cells and reagents. Human embryonic kidney (HEK-293T) cells were maintained in DMEM (Gibco) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Stably transfected HEK-293T cells were maintained in the above DMEM medium with addition of 200 µg/ml G418. The reagents used in the study were purchased as follows: pGenesil-2 plasmid from Wuhan Genesil Biotech Co., Ltd; Rabbit polyclonal antibody to p-ERK1/2 and Rabbit polyclonal to total-ERK1/2 from Santa Cruz Biotech, Inc; Fluo-3/AM, Ionomycin and G418 from Sigma Co., Ltd; Recombinant Human EGF and goat anti-rabbit IgG from Jingmei Biotech Co., Ltd; Lipofectamine 2000, Fetal bovine serum (FCS) from Invitrogen Co., Ltd; Bay K8644 from Alexis; One step Perfect Real Time PCR Kit and Trizol reagents from TaKaRa Biotech Co., Ltd.

Construction of shRNA vectors. Two shRNAs targeting Pkhd1 mRNA (GI: 25777664) were designed according to the standard selection criteria (Reynolds et al., 2004). Double-stranded small hairpin RNA (shRNAs) and control shRNA were synthesized (Table 1) and then were cloned into pGenesil-2 vector (Fig. 1).

Establishment of Pkhd1-silenced cell lines. According to the manufacturer’s protocol for Lipofectamine 2000, vectors with Pkhd1 shRNA, HK-A and pGenesil-2 plasmid were transfected into subconfluent HEK-293T cells for 24 h, 48 h. Expression of Pkhd1 mRNA was measured by quantitative PCR. The vector with the least Pkhd1 mRNA level was selected to establish the stable Pkhd1-silenced cell lines. In parallel, we used pGenesil-2 plasmid and HK-A shRNA vector to transfect HEK-293T cells to produce respective stable control cell lines. After transfection, G418 (600 µg/ml) selection was commenced and maintained for a week to obtain the G418-resistant clones. The selected cells were then resuspended and seeded in 100-mm culture plates with a cell density of 1 \times 10^5 per plate. The G418-resistant single colonies were picked and transferred onto 24-well plates and the stably transfected HEK-293T cells were propagated in the continual presence of G418 (200 µg/ml).

RNA isolation and real time PCR. Total RNA was isolated from the transient or stable transfected HEK-293T cell lines using Trizol reagents according to the manufacturer’s instructions. Real-time PCR was performed with the LightCycler system (Roche). The following oligonucleotide primers and Taqman probe were used: Pkhd1-FP, 5’-GCCACCAATGGAAGATCTTGA-3’; Pkhd1-RP, 5’-ACGGCCAAAGGATGTTGCA-3’; Pkhd1-Taqman Probe, FAM-ACGCCCATATTGGAAGATGTTGCA-TAMRA; Human β-actin-FP, 5’-CTTCGGCCACCCAACACATC-3’; Human β-actin-RP, 5’-CTTGGCACTCCATCCCTGGA-3’; Human β-actin Taqman Probe; FAM-ATCAAGATCATGGCTCCCTGAGCGC-TAMRA.

Cell proliferation assays. Cells were seeded onto 96-well plate in DMEM with 10% FBS and 100 µg/ml penicillin/streptomycin for 24 h and treated with EGF, vaneparil, or Bay K8644. Cell proliferation rates were determined by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay method.

<table>
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<th>Table 1. shRNAs against Human Pkhd1</th>
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<td><strong>shRNA names</strong></td>
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<td>PKHD1 shRNA1</td>
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<td>PKHD1 shRNA2</td>
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<td>HK-A(control shRNA)</td>
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Western blotting. The HEK-293T cells were washed twice with phosphate-buffered saline (PBS) and lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1% NP-40) with protease inhibitors (0.1 mg/ml aprotinin, 5 µg/ml leupeptin, 50 µg/ml pepstatin, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF) for 30 min on ice. The lysates were centrifuged at 12,000 g for 10 min at 4°C. Supernatant was collected and stored at −20°C for use. 20 µg total protein was run on 12% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in tris-buffered saline tween-20 (TBS-T) with 3% BSA for an hour at room temperature. After that, they were incubated with the primary antibodies for overnight at 4°C and followed by incubation with the goat-anti rabbit HRP-conjugated secondary antibodies for an hour at room temperature. Immunoreactive bands were identified using SuperSignal west pico chemiluminescent substrate (Pierce) and exposed to X-rays films (Kodak).

Measurement of intracellular Ca2+. After the cells cultured on 6-well plate in DMEM with 10% FBS reached approximately 80% confluent states, they were rinsed with HEPES buffer for three times and loaded with 2 µM Fluo-3/AM at 37°C for 30 min, and rinsed again. Fluorescence intensity was measured with confocal microscope (Leica TCS SP2) at excitation wavelengths of 488 nm, and the emitted wavelengths of 522 nm at five different locations. At the end of each experiment, cells were permeabilized with HEPES buffer containing 10 µM Ionomycin to determine the Fmax, and then HEPES buffer containing 10 µM Ionomycin and 10 mM EGTA was added to determine the Fmin. The fluorescence intensity was converted to Ca2+ concentration using the equation \[
\left[\text{Ca}^{2+}\right]_i = \frac{K_d \times (F - F_{\text{min}})}{F_{\text{max}} - F},
\] where the dissociation constant \(K_d\) of Fluo-3/AM for Ca2+ is 400 nM, Fmax and Fmin represent fluorescence intensity for Ca2+ -saturating and Ca2+ -free conditions respectively.

Statistical analysis. Data are presented as Means ± SE. T-test was used to compare the data, and \(p < 0.05\) was taken as the level of significance. All results were analyzed by statistical software SPSS11.0.

Results

Establishment and characterization of stable PKHD1-silenced HEK-293T cell lines. As the inhibition of PKHD1 mRNA expression in the cells transfected with shRNA2 vector was much more significant, it was chosen to establish the stable PKHD1-silenced cell lines (Fig. 2A). It was found that the PKHD1 mRNA level was markedly decreased in the gene-silenced cell lines but not in the controls while the PKHD1 mRNA level of the controls were similar to that of wild-type cells (Fig. 2B). These results showed that PKHD1 was down-regulated by the shRNA in the established stable cell lines and the gene silencing is specific.
Hyperproliferative response of \textit{PKHD1}-silenced cell lines to stimulation with EGF. A significant increase of proliferation rates in \textit{PKHD1}-silenced cells was observed after the stimulation with EGF at concentrations of 20 ng/ml, and the proliferation rates of control groups were similar to those of wild-type cells (Fig. 3A). It has been reported that the EGF treatment can induce proliferation by activating ERK1/2 in many cell types (Falin \textit{et al.}, 2005; Liu \textit{et al.}, 2006; Zhuang and Schnellmann, 2004; Yamamoto \textit{et al.}, 2003). In our study, indeed, the EGF treatment increased the level of ERK1/2 phosphorylation in all cell groups with the highest effect in \textit{PKHD1}-silenced cell lines. And PD98059, an inhibitor for ERK1/2 activation, showed inhibitory effect on EGF-induced cell proliferation in all the four cell groups, (Fig. 3B). Thus the results suggest that the hyperproliferative effect of EGF is mediated by ERK1/2 in \textit{PKHD1}-silenced cells.

Fig. 2. The expression of \textit{PKHD1} mRNA after RNA interference. (A) The levels of \textit{PKHD1} mRNA after transfection. From left to right, the wild type, pGenesil-2, HK-A, \textit{PKHD1}shRNA1 and \textit{PKHD1}shRNA2. The mRNA levels of cells transfected with \textit{PKHD1}shRNA1 and \textit{PKHD1}shRNA2 were significantly lower than in the wild-type and HEK-293T transfected with HK-A and pGenesil-2. \textit{PKHD1}-shRNA2 showed the largest amount of inhibition. One asterisk indicates a significant difference at $p < 0.05$ versus HEK-293T. Two asterisk indicates a significant difference at $p < 0.001$ versus HEK-293T. (B) The levels of \textit{PKHD1} mRNA in the stable transfected cell lines. \textit{PKHD1} mRNA level was markedly decreased in the \textit{PKHD1}-silenced cell lines ($p < 0.001$), but not in the control cell lines. The \textit{PKHD1} mRNA level of the controls was similar to that of wild-type HEK-293T cells.

Fig. 3. Proliferation response of \textit{PKHD1}-silenced HEK-293T cells to the EGF stimulation. (A) Four cell groups were treated with EGF (0 ng/ml), EGF (20 ng/ml), PD98059 (20 M), EGF (20 ng/ml) + PD98059 (20 M) respectively for 24 h. Cell proliferation rates were determined by the MTT assay. One asterisk indicates a significant difference at $p < 0.01$ versus control without EGF treatment; triangle indicate $p < 0.01$ versus the HEK-293T cells with EGF treatment. (B) EGF induced ERK1/2 phosphorylation in \textit{PKHD1}-silenced HEK-293T cells. All cell groups treated with EGF show increased ERK1/2 phosphorylation with the highest effect on the \textit{PKHD1}-silenced HEK-293T cells.
PKHD1-silencing decreases the intracellular Ca\textsuperscript{2+} concentration in HEK 293T cells. Since fibrocystin interacts with CAML, a protein that is involved in Ca\textsuperscript{2+} signaling, an altered basal Ca\textsuperscript{2+} level may be expected in PKHD1-silenced cells. In our experiments, the intracellular Ca\textsuperscript{2+} concentration of them was significantly lower than that of wild-type HEK-293T cells (\(p < 0.001\)), but no significant difference between control groups and wild-type cells was observed (Table 2). The results suggest that fibrocystin participates in modulating intracellular Ca\textsuperscript{2+} in epithelial cells of autosomal recessive polycystic kidney.

### Table 2. Intracellular Ca\textsuperscript{2+} Concentration in the silenced HEK-293T Cells and controls

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<th>Groups</th>
<th>Intracellular Ca\textsuperscript{2+} Concentration</th>
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<tbody>
<tr>
<td>HEK-293T</td>
<td>82.1 ± 7.1 nM</td>
</tr>
<tr>
<td>pGenesil-2</td>
<td>80.7 ± 5.5 nM*</td>
</tr>
<tr>
<td>HK-A shRNA</td>
<td>81.2 ± 5.3 nM*</td>
</tr>
<tr>
<td>PKHD1 shRNA</td>
<td>62.4 ± 7.7 nM*</td>
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*: \(p < 0.001\) versus the HEK-293T cells. \(\Delta\): \(p > 0.05\) versus the HEK-293T cells.

EGF-induced cell excessive proliferation is caused by decreased intracellular Ca\textsuperscript{2+} concentration. To determine whether EGF-induced excessive cell proliferation was caused by change of calcium level, HEK-293T cells were incubated in 1 \(\mu\)M verapamil for 0, 1, 2, 4, 8, 16, 24 h. Then the cells were loaded with Fluo-3/AM and the intracellular calcium was measured. As shown in Fig. 4A, verapamil treatment caused a stable decrease of intracellular calcium after 1 h and this was dose-dependent (Fig. 4B). When cells were treated with verapamil for 8 h, and then incubated in medium with 20 ng EGF for 24 h, the cell proliferation rates were significantly increased (Fig. 4C). Also, the verapamil treatment increased the level of activated ERK1/2 in EGF-treated cells (Fig. 4D). The results imply that decrease of the intracellular Ca\textsuperscript{2+} concentration in wild-type cells by a calcium channel blocker was sufficient for EGF-induced cell overproliferation through stimulating ERK1/2 overactivation.

**Fig. 4.** Decreased intracellular Ca\textsuperscript{2+} concentration caused EGF-induced cell hyperproliferation. (A) Verapamil decreases the intracellular Ca\textsuperscript{2+} concentration of HEK-293T cells after 1 h. Intracellular Ca\textsuperscript{2+} concentration was measured after cells were incubated in 1 \(\mu\)M verapamil for 0, 1, 2, 4, 8, 24 h. (B) Verapamil decreases intracellular Ca\textsuperscript{2+} concentration of HEK-293T cells in dose-dependent. Intracellular Ca\textsuperscript{2+} concentration was measured after incubation of 0, 0.5, 1, 2, 4 \(\mu\)M verapamil for 4 h. (C) Verapamil increases EGF-induced cell proliferation. After cells treated with 1 \(\mu\)M verapamil and PD98059 for 4 h they were incubated in medium with 20 ng EGF for 24 h and the cell proliferation rates were measured. Corresponding control groups were set up. Two asterisks indicate a significant difference at \(p < 0.01\) versus HEK-293T with EGF treatment. (D) Verapamil increases EGF-induced ERK1/2 activation. Cells were treated with 1 \(\mu\)M verapamil and PD98059 for 4 h. Then they were incubated in medium with 20 ng EGF for 1 h. Corresponding control groups were set up.
Elevation of intracellular Ca$^{2+}$ inhibits the EGF-dependent excessive proliferation of \textit{PKHD1}-silenced cells. If the mechanism of proliferation by EGF stimulation in \textit{PKHD1}-silenced cells is a consequence of disturbed intracellular Ca$^{2+}$ homeostasis, then the elevation of intracellular Ca$^{2+}$ should inhibit the EGF-dependent excessive proliferation of \textit{PKHD1}-silenced cells. To prove this, we measured the proliferation rates of \textit{PKHD1}-silenced cells treated in EGF alone or in combination with Bay K8644, activator of L-type Ca$^{2+}$ channels. As the results, a dose-dependent increase of intracellular Ca$^{2+}$ was observed in \textit{PKHD1}-silenced cells treated by Bay K8644 (Fig. 5A). The increase of intracellular Ca$^{2+}$ by Bay K8644 showed no effect on the basal proliferation rates but repressed the proliferative response to EGF (Fig. 5B). Thus, the elevation of intracellular Ca$^{2+}$ can repress EGF-dependent excessive proliferation of \textit{PKHD1}-silenced cells.

To explore the relationship between intracellular Ca$^{2+}$ and EGF-induced ERK1/2 activation, level of phosphorylated ERK were measured after Bay K8644 treatment. As the result, ERK1/2 overactivation was repressed by Bay K8644 (Fig. 5C). This suggests that elevation of intracellular Ca$^{2+}$ can inhibit the EGF-dependent excessive proliferation of \textit{PKHD1}-silenced cells though repressing ERK1/2 activation.

**Discussion**

Fibrocystin is deduced to be a putative membrane receptor-like protein that may involve in ligand-binding, cell-cell, and cell-matrix interactions (Ward et al., 2002; Onuchic et al., 2002). To date, however there have been few studies on the function of the large complex protein. Present study aimed to investigate the effect of fibrocystin on regulation of intracellular Ca$^{2+}$ and EGF-induced MAPK signaling. It was found that disturbed intracellular Ca$^{2+}$ homeostasis due to knock-down of \textit{PKHD1} caused EGF-induced cell overproliferation in the \textit{PKHD1}-silenced cell though increasing ERK1/2 activation.

Fibrocystin is localized to the cilium of renal epithelial cells which plays a role of sensor of mechanical and chemical stimulation from the lumen of renal tubules (Ward et al., 2003). For instance, \textit{in vitro} bending the cilium initiates a transient increase in intracellular Ca$^{2+}$ and loss of cilia abolishes the response of the renal epithelium to fluid flow (Praetorius and Spring, 2001; Praetorius and Spring, 2003). \textit{In vivo}, mutation of the \textit{Tg737}, the gene encoding cilia-associated proteins, leads to attenuation of mechano-regulation of intracellular Ca$^{2+}$ (Liu et al., 2005). Apparently, the misregulation of intracellular Ca$^{2+}$ plays a key role in the pathogenesis of
and ERK1/2 activation were increased in HEK-293T cells overactivity in HEK-293T cell treated with EGF. overactivity is a feature of cystic tubules in both dominant and recessive polycystic kidney disease (Kato, Y., Tapping, R. I., Huang, S., Watson, M. H., Ulevitch, R. J. et al. 1998). And inhibition of EGFR slows down the disease progression in animal with ARPKD (Sweeney, Jr. et al., 2000; Sweeney, Jr. et al., 2003). Treatment with cyst fluid increased the proliferation of cells from cystic collectine tubule. Meanwhile, cyst fluid deprived of EGF decreased the proliferate rate (MacRae, D. K., Nemo, R., Sweeney, W. E. and Jr. and Avner, E. 2000). The EGF-induced hyperproliferation and hyperproliferative and ERK1/2 overactive response to EGF in ARPKD patients, significant lower basic Ca\(^{2+}\) concentration was observed in cystic cells compared with normal kidney cell (Yamaguchi et al., 2006). In present study, we found that inhibition of \(PKHD1\) by RNA interference led to lowered intracellular Ca\(^{2+}\) concentration in the HEK-293T cells.

Proliferation and differentiation of epithelial cell are regulated by various cytokines and growth factors including EGF. EGF induces cell proliferation in a variety of cell types by binding to a prototype transmembrane tyrosine kinase receptor to activate ERK1/2 (Kato et al., 1998). The EGF/EGFR axis overactivity is a feature of cystic tubules in both dominant and recessive polycystic kidney disease (Kato et al., 1998). And inhibition of EGFR slows down the disease progression in animal with ARPKD (Sweeney et al., 2000; Sweeney, Jr. et al., 2003). Treatment with cyst fluid increased the proliferation of cells from cystic collectine tubule. Meanwhile, cyst fluid deprived of EGF decreased the proliferate rate (MacRae et al., 2004). It has been reported that phosphorylated ERK1/2 of cystic kidneys is much higher than that of noncystic kidney (Veizis and Coton, 2005). These imply that EGF and ERK1/2 play an important role in the development of the cystic formation. In our study, \(PKHD1\)-silencing caused hyperproliferation and ERK1/2 overactivity in HEK-293T cell treated with EGF. Our experiment also showed that the EGF-induced proliferation and ERK1/2 activation were increased in HEK-293T cells with Ca\(^{2+}\) channel blocker pre-treatment. In contrast, elevation of intracellular Ca\(^{2+}\) by Ca\(^{2+}\) channel agonist reversed the hyperproliferative and ERK1/2 overactive response to EGF in \(PKHD1\)-silenced cells. Combined together, these data emphasize the role of Ca\(^{2+}\) regulation in pathogenesis of ARPKD and support that the fibrocystin may act as a membrane receptor participating in Ca\(^{2+}\) signaling. Thus, it may be postulated that the lowered intracellular Ca\(^{2+}\) leads to EGF-induced hyperproliferation in cells with silenced \(PKHD1\) and the loss of fibrocystin by the gene mutation causes hyperproliferation of kidney epithelial cells and cyst formation through change of intracellular Ca\(^{2+}\) concentration in ARPKD.

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References


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